EXHIBIT 2

Experimental Report

METHODS

Animals

Adult male Sprague-Dawley rats were purchased from the Animal Resources Centre (ARC), Perth, Australia, and the Herston Medical Research Centre, The University of Queensland. Rats were housed in a temperature controlled environment (21 ± 2° C) with a 12h/12h light/dark cycle. Food and water were available *ad libitum*. Ethical approval for this study was obtained from the Animal Experimentation Ethics Committee of The University of Queensland.

Reagents and materials

Isoflurane (Forthane®) was obtained from Abbott Australasia Pty Ltd (Sydney, Australia). Sodium benzylpenicillin vials (600 mg) were purchased from CSL Ltd (Melbourne, Australia). Normal saline ampoules were obtained from Delta West Pty Ltd (Perth, Australia) and heparinised saline (50 IU/5 ml) was purchased from Astra Pharmaceuticals Pty Ltd (Sydney, Australia).

Single lumen polyethylene tubing (I.D. 0.2 mm, O.D. 0.6 mm for i.t. studies and I.D. 0.5 mm, O.D. 1.00 mm for i.v. studies) was purchased from Auburn Plastics and Engineering Pty Ltd (Sydney, Australia). Sterile siliconized silk sutures (Dysilk™) were obtained from Dynek Pty Ltd (Adelaide, South Australia) and Michel clips were purchased from Medical and Surgical Requisites Pty Ltd (Brisbane, Australia).

Chronic Constriction Injury (CCI) of the Sciatic Nerve

Rats were anaesthetised with ketamine (80 mg/kg) and xylazine (8 mg/kg) administered by intraperitoneal injection, and a chronic constriction injury (CCI) of the sciatic nerve was produced according to the method of Bennett and Xie (1988). Briefly, the left common sciatic nerve was exposed at mid-thigh level by blunt dissection through the biceps femoris. Proximal to the trifurcation, ≈ 10 mm of nerve was freed of adhering tissue and four loose ligatures (3.0 silk) were tied around the sciatic nerve (≈ 1 mm apart). The incision was closed in layers. After surgery, rats

received benzylpenicillin (60 mg s.c.) to prevent infection and were kept warm during surgical recovery. Rats were housed singly for 14 days prior to opioid or vehicle administration.

Rats were inspected daily from the time of CCI-surgery with regard to posture of the affected hindpaw, exploring behaviour, body weight and water intake, and any signs of autotomy. Early signs of autotomy were seen in one rat (gnawing of claw tips and some surrounding tissue on the ipsalteral hindpaw) and this animal was promptly euthanased.

Surgery

Intrathecal Catheter Insertion

Ten to eleven days post CCI-surgery of approximately seven days after the induction of hindpaw inflammation, rats were deeply anaesthetised with a mixture of ketamine (80 mg kg^{-1}) and xylazine (8 mg kg^{-1}) administered as a single intraperitoneal (i.p.) injection. Prior to surgery, the back and neck regions of the rat were shaved and the skin cleansed with betadine surgical scrub. The rat was then placed in a prone position and the L6 lumbar vertebra was located by palpation of the tuber sacrales of the os ileum (Hebel & Stromberg 1976). A 6 cm incision was made in the midline of the back, 3 cm caudal and 3 cm cephalad to L6. A subcutaneous pocket (for the intrathecal catheter) was formed by blunt dissection with scissors on both sides of the incision. The fascia covering the superficial muscles of the back were cut in a 5 mm V-shaped incision that encompassed L5. Additional 5 mm caudal incisions were made parallel to L6. The fascia was then retracted and the lumbar muscles surrounding the base of L5 and L6 were removed, as was the m. interspinalis between the spinous processes of L5-L6.

Following removal of the L6 spinous processes with rongeurs, the soft tissue beneath the L5 iliac arch was removed, exposing the dura mater. The dural membrane was pierced with a 23G needle, releasing clear CSF. A polyethylene catheter (O.D. 0.6 mm, I.D. 0.2 mm; 20 or 50 cm length for acute and chronic experiments respectively) pre-filled with saline, was carefully advanced a distance of 1 cm

into the intrathecal space and a small volume of saline (20 μL) was administered through the catheter. If leakage of saline around the catheter was observed, the rat was excluded from further experimentation. After successful completion of the 'leak test', the intrathecal (i.t.) catheter was fixed with dental cement onto the surrounding muscle and skin ≈ 2 cm from L5, exteriorised through a subcutaneous (s.c.) tunnel to a small incision at the base of the neck and sutured in position. After suturing of the lumbar muscles and skin, rats received benzylpenicillin (50000 IU i.p.) and enrofloxacin (5 $mgkg^{-1}$ s.c.) to prevent infection and were kept warm during recovery from anaesthesia. Following completion of the surgery, rats were housed singly for a recovery period of 5-7 days prior to i.t. drug administration. On the day following surgery, the local anaesthetic, lignocaine (2%, 20 μL) was administered via the i.t. catheter. If complete paralysis of both hind legs was not observed, rats were excluded from further experimentation.

Drugs Administered

- Mr1A: a stock solution was supplied in a concentrations of 1 mg/mL. This solution was used to produce the two doses administered to CCI-rats by the i.t. route.
- Morphine: morphine hydrochloride powder (B.P.) was purchased from the Royal Brisbane Hospital Pharmacy (Brisbane, Australia). Morphine hydrochloride was dissolved in isotonic saline to produce the desired concentration for i.t. administration.

Storage of Stock Solutions of Peptide

An aliquot (10 μL) of the stock solution of the peptide was stored at –20°C prior to use for animal experimentation. Immediately prior to experimentation, the aliquot of the peptide was thawed at room temperature and then diluted to the required concentration with sterile saline to achieve the desired final peptide concentration for subsequent i.t. administration. Unused portions of thawed peptide stock solution were discarded to waste to ensure that the peptide only underwent 1 freeze-thaw cycle.

Intrathecal Drug Dosing

Peptide

Drug-naïve CCI-rats: acute studies

On day 14 post-CCI surgery, individual groups of drug-naïve-CCI rats received an i.t. bolus injection of Mr1A, in a volume of 10 μL. Antinociception was assessed using von Frey filaments (see below for details) over a 3 h post-dosing interval. In a separate experiment drug-naive CCI rats received one bolus injection of morphine (3.5-50nmol) in a volume of 10μL. Similarly, drug-naïve, control CCI rats also received bolus injections of saline (10μL) by the i.t. route on day 14 post CCI surgery.

I.t. injections were followed by a saline flush (20 μL) to ensure complete drug delivery.

Assessment of Antinociception: CCI rats

Von Frey Filaments

Tactile allodynia, the distinguishing feature of neuropathic pain, was quantified using Von Frey filaments. Rats were transferred to wire mesh testing cages (20 cm x 20 cm x 20 cm) and allowed to acclimatise for 10 min. Von Frey filaments were used to determine the lowest mechanical threshold required for a brisk paw withdrawal reflex. Briefly, starting with the Von Frey filament that produced the lowest force, the filament was applied to the plantar surface of the hindpaw until the filament buckled slightly. Absence of a response after 5 s prompted use of the next filament of increasing weight. Filaments used produced a buckling weight of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 g and these were calibrated regularly. A score of 20 g was given to animals that did not respond to any of the Von Frey filaments.

Side-effects

Side-effects were recorded in a semi-quantitative descriptive manner.

Verification of correct i.t. catheter placement

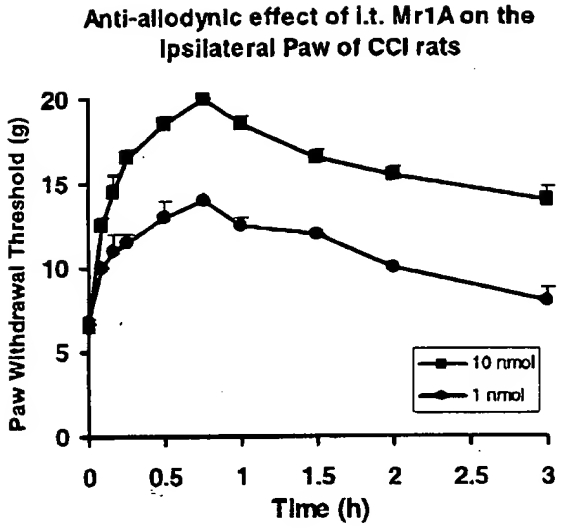
At the completion of each experiment, malachite green dye (30 μL) was injected via the i.t. catheter whilst rats were lightly anaesthetized with O_2:CO_2 (50%:50%). Thirty seconds later, rats were decapitated and the spinal column was exposed surgically. Data from rats where there was evidence of subcutaneous dye leakage at the site where the catheter entered the back muscles above L6 or failure of the dye to distribute at least 3-4 cm along the spinal cord, were excluded from the analysis.

Data Analysis

The areas under the degree of antinociception (expressed as the percentage maximum effect, %MPE) versus time curves (AUC values) for the peptide was calculated from time = 0 to 3 h. A dose-response curve for the peptide was constructed by plotting AUC values versus the i.t. peptide dose (expressed in nmol).

RESULTS

1A



1B

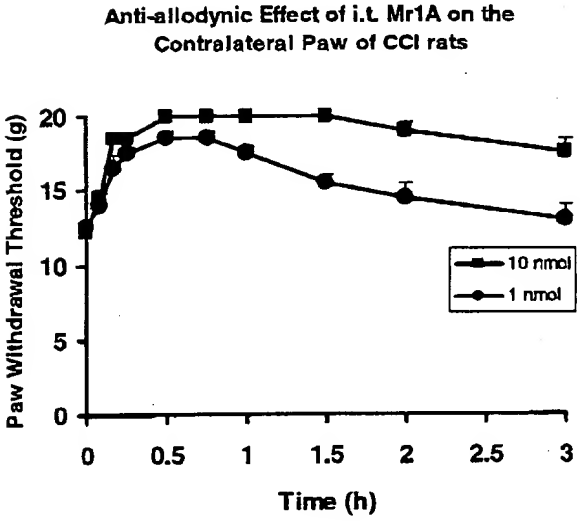


Fig 1

(A) Mean (± SEM) paw withdrawal threshold versus time curve evoked by i.t. Mr1A (1 and 10 nmol) for the relief of tactile allodynia (hypersensitivity to light pressure) in the ipsilateral hindpaw of rats with a chronic constriction injury (CCI) of the sciatic nerve.

(B) Mean (± SEM) paw withdrawal threshold versus time curve evoked by i.t. Mr1A (1 and 10 nmol) for the relief of tactile allodynia in the contralateral (non-injured side) hindpaw.

Table of side-effects observed following intrathecal administration of Mr1A

Mr1A				
Administered Dose & Route	**Side effects observed**	**Onset**	**Duration**	**Severity** +++ severe ++ moderate + mild - none
1 nmol Intrathecal	hyperactivity	5-15 min	30 min	+
	grooming	5 min	5-15 min	++
	arched back posture	5-15 min	1 h	++
	hypersalivation	15-30 min	1 h	+
	sedation	45 min	1 h	+
10 nmol Intrathecal	hyperactivity	5-15 min	30 min	++
	grooming	5 min	5-30 min	++
	arched back posture	5-15 min	1 h	+++
	sedation	45 min	1-2 h	+

N.B. Grooming behaviour was observed for 5-10 min after the i.t. administration of saline (see Fig 2). The human correlate of this behaviour is probably 'itch'. It is possible that the i.t. bolus injection of vehicle resulted in initial histamine release in the spinal cord which quickly dissipated.

The arched back posture and staring behaviour observed are mild neuro-excitatory behaviours that have been observed previously following the intracerebroventricular (icv) administration of morphine-3-glucuronide (M3G), the analgesically inactive major metabolite of morphine, in rats. If the icv dose of M3G is further escalated, rats exhibit myoclonus of the face and limbs, followed by rearing, allodynia, wild running and tonic-clonic convulsions in a dose-dependent manner.

In humans, neuro-excitatory behaviours e.g. myoclonus of the limbs and allodynia, have been reported in some cancer patients following high-dose intrathecal morphine administration. The same cellular mechanism is thought to underpin the neuro-excitatory behaviours evoked by M3G and high-dose intrathecal morphine. This mechanism remains to be defined.

SUMMARY

Mr1A (1 & 10 nmol) given by the i.t. route produced dose-dependent relief of tactile allodynia in rats with a chronic constriction injury of the sciatic nerve.

Overall, the side-effects produced by i.t. Mr1A were very mild in nature when compared with the side-effects (serpentine tail movements, whole body shaking) produced by i.t. doses of MVIIA (ziconotide).

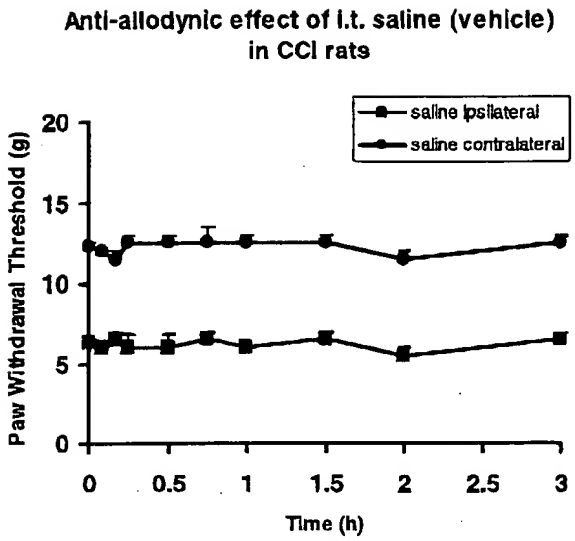


Fig 2

Intrathecally administered saline did not produce significant antinociception in either the ipsilateral or contralateral paws when assessed using (A) a non-noxious mechanical stimulus (von Frey filaments) in CCI rats. The findings indicate that neither the i.t. vehicle (saline), nor the experimental procedures themselves evoke significant antinociception.

Table of side-effects observed following intrathecal and epidural administration of saline (vehicle)

Saline				
Administered Route	**Side effects observed**	**Onset**	**Duration**	**Severity** +++ severe ++ moderate + mild - none
Intrathecal	Grooming	5 min	10 min	++
	↓ Movement (sedation?)	90 min	~1 h	+

The grooming behaviour observed in the first 5-10 min following intrathecal administration of vehicle (saline), shows that this behaviour is at least in part, not drug related. Almost certainly this behaviour is due to transient release of histamine following bolus vehicle injection.

Morphine

Pain Relief

Intrathecal (i.t.) administration of morphine to CCI-rats produced a dose-related increase in the anti-allodynic response for doses in the range 3.5-17 nmol in the ipsilateral hindpaw, together with dose-dependent antinociception in the contralateral hindpaw (Fig 3). However, further increasing the magnitude of the administered i.t. dose of morphine (35 & 50 nmol) did not produce larger anti-allodynic or antinociceptive responses, clearly showing that in CCI-rats, i.t. morphine appears to be only a partial agonist.

When i.t. morphine was administered in a dose of 3.5 nmol to CCI-rats, there was a rapid onset of action with the peak anti-allodynic effect occurring at 0.5 h post-dosing; the duration of action was ≈ 2 -3 h (Fig 4A). Similarly, peak antinociception in the contralateral hindpaw occurred at 0.5 h post-dosing and the corresponding duration of action was ≈ 3 h (Fig 4A).

Administration of i.t. morphine in a dose of 10 nmol to CCI-rats, also produced a rapid increase in the paw withdrawal threshold in the ipsilateral hindpaw such that the peak anti-allodynic effect occurred at 0.25 h and the duration of action was > 3 h. In the contralateral hindpaw, peak antinociception occurred at 0.75 h post-dosing and the duration of action was = 3 h (Fig 4B).

Increasing the magnitude of the i.t. dose of morphine from 10 to 17 nmol resulted in a rapid onset of the anti-allodynic effect in the ipsilateral hindpaw with the peak effect occurring at 0.75 h; the corresponding duration of action was > 3 h. Additionally, the onset of antinociception, time of peak effect and the duration of antinociception in the contralateral hindpaw mirrored the time course of the anti-allodynic effects observed in the ipsilateral hindpaw.

Although the 17 nmol dose of i.t. morphine produced a sub-maximal anti-allodynic effect, an approximate doubling of the i.t. morphine dose to 35 nmol did not produce a further increase in the magnitude or the duration of either the anti-allodynic response in the ipsilateral hindpaw or the antinociceptive response in the contralateral hindpaw. Furthermore, when the magnitude of the i.t. morphine dose was increased to 50 nmol, the extent and duration of the anti-allodynic response in the ipsilateral hindpaw and the antinociceptive response in the contralateral hindpaw remained at the same sub-maximal level as that produced by i.t. morphine in doses of 17 and 35 nmol.

Thus, it is clear from the foregoing that in rats with a chronic constriction injury of the sciatic nerve, i.t. morphine is a partial agonist for the alleviation of tactile allodynia in the ipsilateral hindpaw.

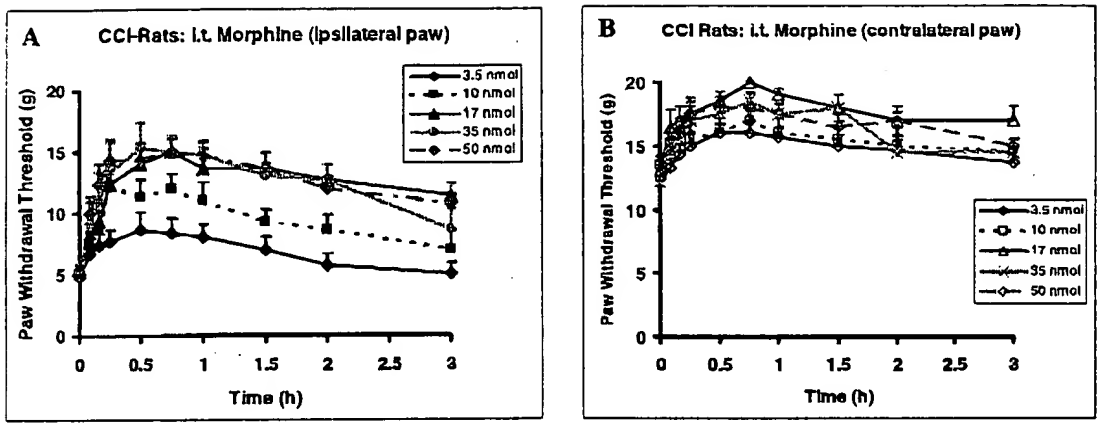
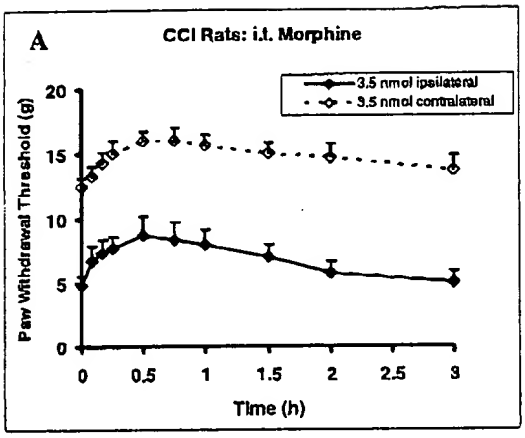
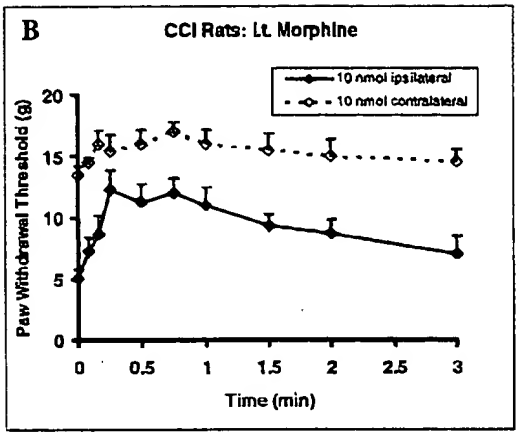


Fig 3 Mean (± SEM) paw withdrawal threshold versus time curves produced by i.t. doses of morphine (3.5 - 50 nmol) in rats with a chronic constriction injury of the left sciatic nerve. Panels A and B show the time course of the anti-allodynic effects of i.t. morphine in the ipsilateral and contralateral hindpaws respectively for the first 3 h post-dosing. Irrespective of the magnitude of the dose administered, there was a rapid onset of the anti-allodynic effect (within 5 min post-dosing) in the ipsilateral hindpaw which was mirrored by a rapid onset of antinociception in the contralateral hindpaw. The mean (± SEM) times to achieve peak levels of anti-allodynia and peak levels of antinociception were similar in the ipsilateral and contralateral hindpaws. Additionally, the mean (± SEM) duration of the anti-allodynic and the antinociceptive effects appeared to be similar in the ipsilateral and contralateral hindpaws for each of the doses investigated. Although there was a dose-dependent increase in the anti-allodynic and antinociceptive responses in the ipsilateral and contralateral hindpaws respectively for doses up to 17 nmol, further increases in the magnitude of the i.t. morphine dose did not produce any further increase in the pain-relieving responses, despite the fact that the 17 nmol dose produced only sub-maximal effects in each of the ipsilateral and contralateral hindpaws. Taken together, these data clearly indicate that i.t. morphine is a partial agonist for the relief of tactile allodynia in rats with a chronic constriction injury of the sciatic nerve.

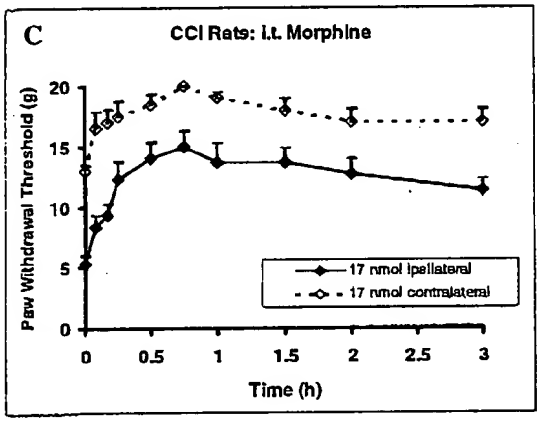
i.t. Morphine (3.5 nmol)



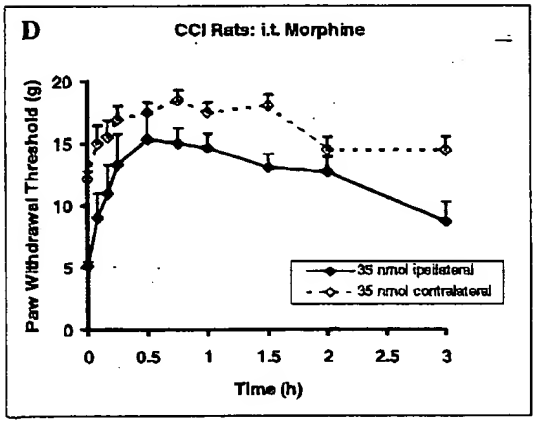
i.t. Morphine (10 nmol)



i.t. Morphine (17 nmol)



i.t. Morphine (35 nmol)



i.t. Morphine (50 nmol)

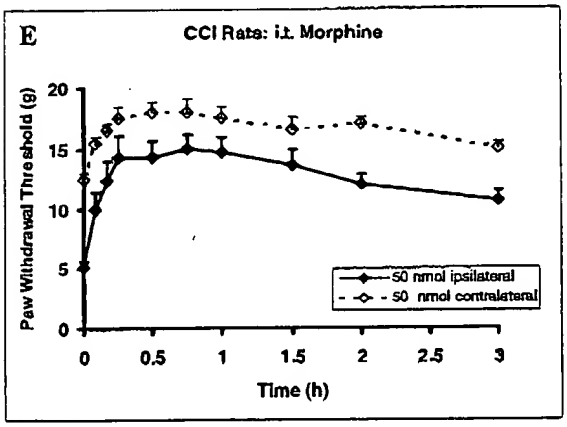


Fig 7 Mean (± SEM) paw withdrawal threshold versus time curves produced by i.t. morphine in doses of 3.5, 10 17, 35 & 50 nmol, are shown in Panels A-E respectively.

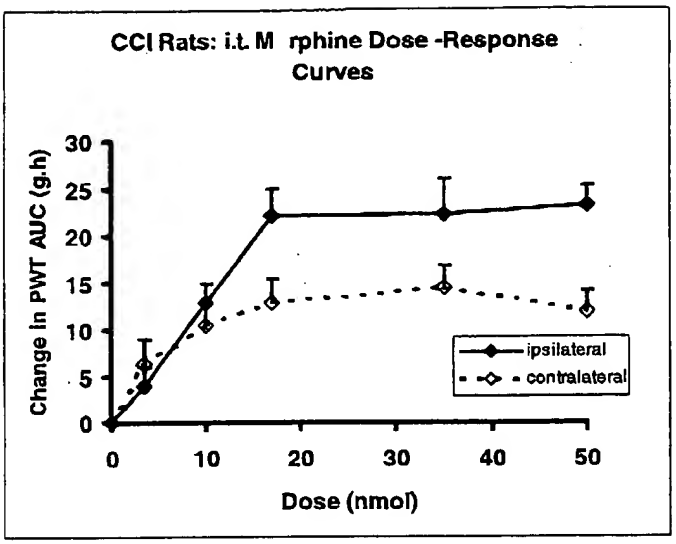


Fig 4 The dose-response curve was produced by plotting the extent and duration of the anti-allodynic or the antinociceptive response (area under the increase in the paw withdrawal threshold above baseline versus time curve) versus the magnitude of the i.t. dose of morphine (expressed in nmol). The corresponding mean (± SEM) ED_{50} dose for the anti-allodynic response in the ipsilateral hindpaw was estimated to be 8.9 (± 1.2) nmol.

Intrathecal Vehicle

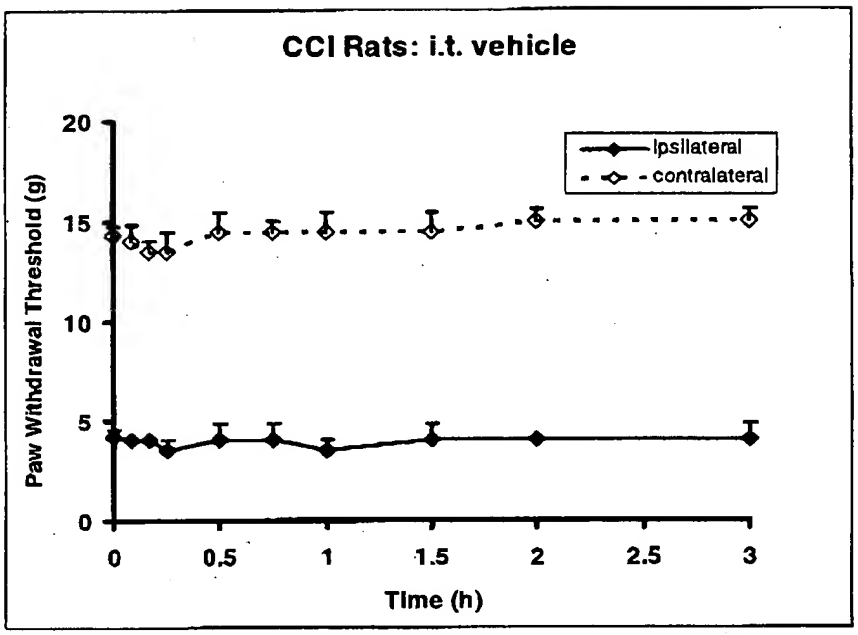


Fig 5 Mean (± SEM) paw withdrawal threshold versus time (hr) following a 10 μL i.t injection of vehicle (5.5 mM sodium acetate buffer, pH 5.5). The absence of an anti-allodynic effect in the ipsilateral hindpaw together with a complete lack of antinociception in the contralateral hindpaw clearly indicate that neither the vehicle nor the experimental procedures themselves contribute to the anti-allodynic or the antinociceptive effects observed following i.t. administration of either morphine or MrIA.